Analysis of the Cross-Reactivity of Various 56 kDa Recombinant Protein Antigens with Serum Samples Collected after *Orientia tsutsugamushi* Infection by ELISA

Chien-Chung Chao,* Erin S. Huber, Terrisita B. Porter,† Zhiwen Zhang, and Wei-Mei Ching

*Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center,
Silver Spring, Maryland; Uniformed Services University of the Health Sciences, Bethesda, Maryland*

Abstract. *Orientia tsutsugamushi*, the etiologic agent of scrub typhus, has a highly expressed and immunodominant 56-kD outer membrane protein. This protein is one of the leading candidates for diagnosis and vaccine development for scrub typhus. Previous studies using recombinant 56-kD protein (r56s) derived from Karp strain (Kpr56) in a mouse model have shown good homologous protection but only moderate to poor heterologous protection. We evaluated the cross-reactivity of recombinant 56-kD proteins from Karp, Kato, Gilliam, TA763, and three chimeric 56-kD proteins. Not all r56s are equally reactive with strain-specific serum samples. These data provide a first glance of how reactive these r56s are toward the antiserum of different strains and which r56 exhibits the broadest reactivity. A formulation of this combination has the potential to provide broad protection against the heterologous challenge and to be used in a highly sensitive diagnostic assay.

INTRODUCTION

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus. The disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and in some cases, disseminated intravascular coagulation that may lead to circulatory failure.[1] It accounts for up to 23% of all febrile illnesses in the Asia-Pacific disease-endemic region.[2] If left untreated, *Orientia* can cause up to 35% mortality. The disease has been re-emerging and occurring in new areas of many countries including Japan, South Korea, Sri Lanka, China, Maldives, India, Palau, Malaysia, Taiwan, and Australia,[3–12] and evidence of antibiotic resistance has been shown.[13,14] At the present time, no vaccine is available for protection against scrub typhus.

Orientia exhibits considerable strain variation.[15–18] Homologous protection developed from natural infection persists for at least one year, but heterologous protection may remain for less than six months.[19,20] Both humoral and cell-mediated immune responses are important in protective immunity against scrub typhus.[21–25] Prior vaccine development efforts using the whole organism have suggested that a scrub typhus vaccine is possible.[19,26,27] Although a recent report suggested that long-term adaptation in egg-yolk sac has increased the yield of *Orientia*,[28] considerable difficulties still exist in mass production of pure *Orientia* and in retaining its stability upon storage. Thus, a subunit vaccine composed of genetically engineered antigens capable of inducing protective immunity in human subjects may be a good alternative.

Western blot analysis of whole cell lysates with scrub typhus patient serum samples has identified at least four protein antigens of *Orientia* with molecular weights of 22 kD, 47 kD, 56 kD, and 110 kD.[29] Among them, the 56-kD antigen accounts for 10–15% of the total cell protein. Almost every clinically diagnosed patient serum recognizes the 56-kD antigen, but not every patient serum reacts with the 22-kD, 47-kD, or 110-kD antigens.[30,31] Recombinant 56-kD protein has been shown to be protective in mice against homologous challenge.[32–34] High titers of antibodies to *Orientia* were also detected in mouse serum samples. A dose-dependent pattern of lymphocyte proliferation and levels of interferon-γ and interleukin-2 production (cytokine profile of Th1 cells) were observed in spleen mononuclear cells from the immunized mice.[32] The 56-kD protein also plays a role in the adhesion and internalization of *Orientia* into host cells, and antibodies against this antigen can block *Orientia* infection of fibroblasts.[24,25,35,36] These results suggest that the 56-kD protein is an ideal candidate for vaccine development.

A recombinant protein (Kpr56) has been shown to provide 60–100% protection against homologous challenge in an outbred mouse model and it was safe and immunogenic in the cynomolgus monkey model.[37] This vaccine candidate has recently been evaluated for protection against heterologous challenge with five non-Karp strains of *Orientia*. Partial protection (11–56%) was observed in CD-1-challenged mice. Administration of a binary vaccine composed of Kpr56 and Ktr56 resulted in similar partial protection (33–67%). A trivalent vaccine (KpKtGm r56) has also been evaluated and showed no significant improvement in heterologous protection. These results demonstrated that the addition of Ktr56 and Gmr56 in vaccine formulation did not provide broader heterologous protection.

Recently, we produced another r56 from the TA763 strain. It has been shown that mouse antibodies against TA763 reacted with many heterologeous strains,[38] and the recombinant TAr56 was recognized by serum samples of many serotypes, demonstrating its broad reactivity. Thus, inclusion of TAr56 may provide additional heterologous protection. To improve the broadness of seroreactivity and to minimize numbers of recombinant proteins included in vaccine, we designed chimeric 1, 2, and 3 proteins by shuffling the epitopes in the variable domains of the Kpr56 and TAr56. We evaluated the seroreactivity of these proteins with serum samples from mice infected with various strains of *Orientia*.

MATERIALS AND METHODS

Chimeric proteins. The 56-kD protein sequences from Karp and TA763 strains of *Orientia* were used as the building blocks for the chimeric proteins. Any substitution of amino

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*Address correspondence to Chien-Chung Chao, Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, 503 Robert Grant Avenue, RM3N71 Silver Spring, MD 20910. E-mail: chien-chung.chao@med.navy.mil
†Deceased.

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14. ABSTRACT
Orientia tsutsugamushi , the etiologic agent of scrub typhus, has a highly expressed and immunodominant 56-kD outer membrane protein. This protein is one of the leading candidates for diagnosis and vaccine development for scrub typhus. Previous studies using recombinant 56-kD protein (r56s) derived from Karp strain (Kpr56) in a mouse model have shown good homologous protection but only moderate to poor heterologous protection. We evaluated the cross-reactivity of recombinant 56-kD proteins from Karp, Kato, Gilliam, TA763, and three chimeric 56-kD proteins. Not all r56s are equally reactive with strain-specific serum samples. These data provide a first glance of how reactive these r56s are toward the antiserum of different strains and which r56 exhibits the broadest reactivity. A formulation of this combination has the potential to provide broad protection against the heterologous challenge and to be used in a highly sensitive diagnostic assay.

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acids was determined based on an epitope prediction program. Chimeric 1 (C1) was designed manually by using the Karp 56-kD protein sequence as the backbone and replacing the variable domain 1 with the TA763 sequence with modifications. The final sequence of C1 is shown in Figure 1A. Similar criteria were used for the design of chimeric 2 (C2), but the sequence of the 56-kD protein from the TA763 strain was used as the backbone, and variable domain 3 was replaced with the sequence of that region from the Karp strain. The sequence is shown in Figure 1B. Chimeric 3 was designed by modifying variable domains 1, 2, and 3 to ensure the presence of epitopes throughout the sequence (Figure 1C). The DNA was synthesized by Bioclone (San Diego, CA) and cloned into a pET29a vector (Novagen, Madison, WI) with built-in *Nde*I and *Xho*I restriction sites. The synthesized DNA sequences were confirmed.

Cloning of 56-kD protein genes from Karp, Kato, Gilliam, and TA763 strains of *O. tsutsugamushi*. The primers with built-in *Bam*H1 and *Nde*I restriction sites for the 56-kD protein gene from amino acids 80 to 456 (according to the sequence of Karp strain) of the open reading frame were designed based on the available DNA sequences of all four strains in the National Center for Biotechnology Information (Bethesda, MD) database. Genomic DNA extracted from renografin gradient–purified *Orientia* was used as template in combination with appropriate primers for each specific strain in a polymerase chain reaction. The amplicons were cloned into a pET24a vector (Novagen). Colonies were selected and sequences of the amplicons were verified. The plasmid was transformed into BL21(DE3) cells (Invitrogen, Carlsbad, CA) for protein expression.

Expression of recombinant 56-kD proteins from BL21(DE3) transformants. BL21(DE3) transformants containing correct

FIGURE 1. Protein sequences of three chimeric proteins of *Orientia tsutsugamuchi*. Variable domains (VD) containing changed amino acids are underlined. **A**, sequence of chimeric 1. Amino acids in red within VD I of TAr56 were replaced with corresponding amino acids in Kpr56. **B**, sequence of chimeric 2. Amino acids in red within VD III of Kpr56 were replaced with corresponding amino acids in TAr56. **C**, sequence of chimeric 3. Amino acids in VD I and VD III were the same as those in chimeric 1 and 2, respectively. Amino acids in VD II of TAr56 were replaced with corresponding amino acids in Kpr56.

amplicons were selected, grown in Luiria-Bertani medium in the presence of 50 µg/mL of kanamycin (Invitrogen) in a 37°C shaker and agitated at 200 rpm. The cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO) when the optical density at 600 nm reached 0.8–1.0. After induction for 19 hours, the cells were centrifuged at 4,000 rpm for 30 minutes in a SS34 rotor to separate cells from medium. The cell pellet was stored at –20°C until use.

Extraction and solubilization of inclusion bodies containing recombinant 56-kD (r56) proteins. The cell pellet was thoroughly resuspended in 2% deoxycholate (Sigma) in 20 mM Tris-HCl, pH 7.5 (buffer A) and sonicated (Sonicator Ultrasonic Liquid Procesor Model XL2020; Misonix Inc., Farmingdale, NY) with a standard tapered microtip) on ice. Disrupted cell extract was centrifuged at $8,000 \times g$ for 30 minutes at 4°C. The pellet was resuspended in 2 M urea (Arcos Organics USA, Morris Plains, NJ) in buffer A, incubated with gentle rocking for 30 minutes, and centrifuged again as described. The entire process was then repeated with 4 M urea in buffer A and then with 6 M urea in buffer A. Finally, the pellet was dissolved and incubated with gentle rocking for 30 minutes in 8 M urea in buffer A. The supernatant (approximately 10 mL) containing most r56 proteins was in 6 M urea.[5]

Chromatographic purification of recombinant 56-kD proteins in 6 M urea. The 6 M urea supernatant containing r56 proteins was purified by diethylaminoethane (DEAE) anion-exchange chromatography with a 50-minute linear gradient of 0.30–0.70 M NaCl (Sigma) in 6 M urea, 20 mM Tris-HCl, pH 8.0 (buffer B). The purity of protein in each fraction was accessed by sodium dodecyl sulfate–polyacrylmide gel electrophoresis, and fractions with > 95% purity were pooled. If the purity of protein was not satisfactory, a second run of DEAE chromatography was carried out after dialysis of the pooled fractions to remove NaCl. The procedure for the second DEAE purification was similar to that for the first DEAE purification but the linear gradient was 0.30–0.60 M NaCl for 50 minutes. The final fractions containing r56 proteins were evaluated for protein purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[5]

Refolding of pure recombinant 56-kD proteins by dialysis. The purified r56 in 6 M urea was refolded by sequential dialysis in decreasing concentrations of urea as described by Ching and others.[5] The purified polypeptides at a concentration of approximately 0.5 mg/mL in buffer B were transferred into a dialysis bag (24 mm, molecular weight cutoff value = 12,000 daltons) and dialyzed sequentially against 4 M, 2 M, and 0 M urea in buffer A for 30 minutes twice at each concentration of urea with gentle stirring. All dialysis procedures except the last step without urea were conducted at room temperature. Usually 3 dialysis bags, with 10 or 20 mL each in a 500-mL or 1,000-mL beaker, respectively, were dialyzed against buffer at a ratio of 1:15. The dialysis was continued overnight without urea in large excess of buffer A at 4°C to remove traces of urea.

Analysis of purified recombinant 56-kD protein and verification of reactivity with antibody by Western blotting. N-terminal sequencing and Western blotting were used to confirm that purified r56 was reactive with antibody. The protein was transferred from the gel onto a 0.45-µm polyvinylidene difluoride (Invitrogen) membrane and stained with CodeBlue staining solution to visualize protein bands. The single band between 37 kD and 50 kD was cut out of the membrane and analyzed

by a protein N-terminal 491 sequencer (Applied Biosystems, Forster City, CA) to confirm that the expressed protein was correct. In addition, another gel was run and transferred onto nitrocellulose membrane. The membrane was blocked with 10% milk in Tris-buffered saline (TBS)–Tween20 (TBST; Bio-Rad, Hercules, CA) for one hour with gentle agitation, washed three times with TBST (five minutes/wash), and incubated with anti-Kpr56 mouse serum in TBST containing 5% milk for one hour at room temperature. The membrane was washed three times (five minutes/wash) and incubated with horseradish peroxidase (HRP)–conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST with 5% milk for one hour. The membrane was then washed and incubated with substrate as described by the manufacturer (Bio-Rad).

Mouse immunization. Female CD-1 mice were intraperitoneally challenged with different strains of live *Orientia* as described.[39] The challenged mice were monitored once a day for an additional 21 days to assess morbidity and mortality. Mice that survived the infection after 21 days were killed three weeks after challenge and their blood was collected by cardiac puncture. The animal protocol used in this study was approved by the Walter Reed Army Institute/Naval Medical Research Center Institutional Animal Care and Use Committee under protocol # D01-06.

Analysis of reactivity of various recombinant 56-kD proteins and serum samples from mice challenged by various strains of *O. tsutsugamushi*. The concentration of each r56 protein antigen was determined by using the Bradford method. The plates were coated with 0.3 μg/well/100 μL of individual protein overnight at 4°C. The experiment was performed as described.[40] Briefly, coated plates were first rinsed three times with 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20 (1× PBST), blocked with 200 μL/well of 10% milk in 1× PBST, and incubated for one hour at room temperature. Primary antibody (mouse serum samples for 12 strains and human serum samples for 2 strains) was serial diluted by a factor of 4 (1:100, 1:400, 1:1,600) in 5% milk in 1× PBST. Plates were washed after blocking and incubated with diluted primary antibody. The plates were then washed again and incubated with HRP-conjugated goat anti-mouse IgG or HRP-conjugated rabbit anti-human IgG (Santa Cruz Biotechnology) at a 1:4,000 dilution for one hour at room temperature. At the end of incubation, plates were washed and substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added. The plates were incubated at room temperature for 15–30 minutes in the dark and read at 405–650 nm on a UVmax kinetic microplate reader (Molecular Devices, Sunnyvale, CA). The mean of the negative controls plus two times the standard deviation was used as the cutoff value for determining a positive result. The cutoff value was 0.1 for mouse serum samples, 0.1 for MAK119 serum samples, and 0.04 for MAK243 serum samples.

RESULTS

Protein purification and Western blotting. All r56 proteins were purified to achieve > 98% purity as shown in Figure 2A. In most cases, protein was overloaded in each gel lane to ensure purity. The same amount of each r56 was loaded onto a gel for Western blotting. Figure 2B shows that all r56 proteins reacted with the specific antibody against the 56-kD
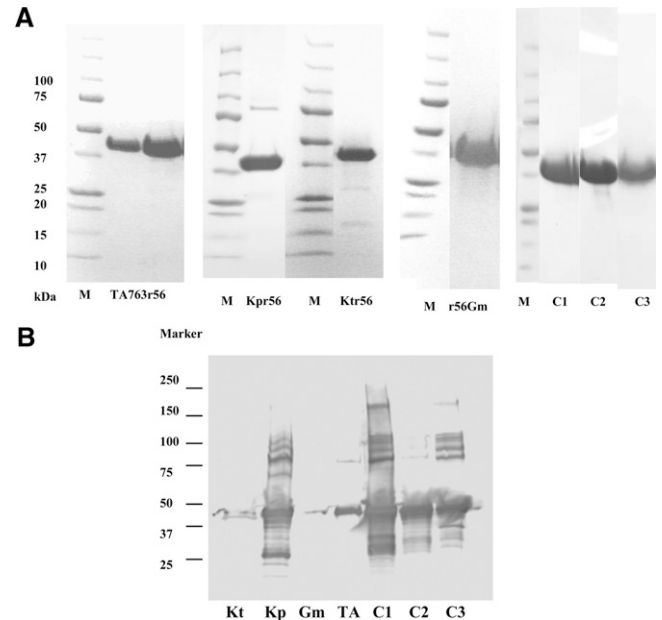

FIGURE 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of all r56 recombinant proteins of *Orientia tsutsugamuchi*. **A**, SDS-PAGE of all 7 r56s with purity > 98%. **B**, Western blot results with anti-Kpr56 mouse serum. All r56s were loaded in equal amounts. This figure appears in color at www.ajtmh.org.

protein from the Karp strain. Although they are all reactive to the same antibody, the intensity of the band differed, indicating that there are differences among different r56. Ktr56 and Gmr56 appeared to be the least reactive with the antibody.

Evaluation of cross-reactivity of r56s with strain-specific mouse serum by enzyme-linked immunosorbent assay. We have repetitively evaluated the protective efficacy of r56 as a vaccine candidate by challenging immunized mice with homologous and heterologous strains of *Orientia*. From our previous work, it is evident that r56 from several different strains when used alone or in combination was unable to provide substantial protection from heterologous challenges. To better understand whether inclusion of certain r56s would be beneficial as a vaccine candidate, we designed three additional chimeric r56 proteins and evaluated their cross-reactivity.

Comparison of cross-reactivity was also performed with r56 protein from Karp, Kato, Gilliam and TA763 strains to determine which r56 appeared to be the most reactive with different strain-specific mouse serum samples. Because mouse serum samples were obtained from mice that survived the lethal challenge by individual strains of *Orientia*, the antibodies generated by each strain should exhibit the strongest reactivity with its respective r56 protein. This feature enabled us to find the minimum number of r56 proteins to provide the broadest and strongest cross-reactivity.

We obtained serum samples from mice that survived challenge with 12 different strains and serum samples from two human patients who were infected with two additional strains. These strains represented a wide range of strain diversity (Table 1) and were carefully selected for the development of a broadly protective vaccine. With the exception of TA763, they were human isolates from a wide range of geographic locations within the *Orientia* triangle. The restriction fragment length polymorphism of *GroEL* and Western blot analysis of

Geographic distribution of different *Orientia tsutsugamushi* strains

Location	Strain name
Malaysia	18032460, 18032404
Thailand	TH-1811, TH1812, TH1814, AFC-27, TA763
Japan	Kato
Burma	Gilliam
New Guinea	Karp
Taiwan	MAK119, MAK243
Australia	Woods, Citrano

whole cell lysate by monoclonal antibody are distinctly different. Their growth rates in tissue culture are also different. Immunization using r56 was never protective to subsequent challenged with MAK119 and MAK243 in our mouse model. Thus, human patient serum samples were used for these two strains.

All serum samples tested diluted to 1:100 reacted with every r56. However, the titers of each serum sample against all seven r56 proteins were different (Tables 2 and 3). Kpr56, Ktr56, Gmr56, and TAr56 had the highest titers with respective serum samples from homologous challenge, confirming that these antigens were most reactive towards their own specific antiserum. It was observed (Table 2) that 1) Kpr56 was the most reactive antigen for serum samples from Th1812, 18032460, 18032404, Woods, and Citrano; 2) TAr56 was the most reactive antigen for TH1811, TH1812, TH1814, AFC-27, 18032404, Woods, Citrano, and MAK243; 3) Gmr56 was the least reactive antigen for serum samples from Karp, Kato, TA763, TH1811, TH1812, AFC27, Citrano, MAK 119, and MAK243; and 4) Ktr56 was the least reactive antigen for serum samples from Karp, Gilliam, TA763, TH1811, TH1814, 18032460, 18032404, Woods, Citrano, MAK119, and MAK243. The results that Ktr56 and Gmr56 were the least reactive with Karp-challenged serum are consistent with Western blot results.

All chimeric r56s reacted similarly for any given serum sample. Ktr56 or Gmr56 was not more reactive than any of

TABLE 2
ELISA titers of 14 strain-specific serum samples against Kpr56, Ktr56, Gmr56, and TAr56 proteins of *Orientia tsutsugamuchi**

Strain	Antigen used			
	Kpr56	Ktr56	Gmr56	TA763r56
Karp	25,600†	< 1,600	1,600	1,600
Kato	1,600	25,600‡	< 1,600	1,600
Gilliam	6,400	1,600	102,400§	25,600
TA763	25,600	6,400	6,400	102,400¶
TH1811	6,400	1,600	1,600	25.600¶
TH1812	6,400	6,400	< 1,600	25,600¶
TH1814	25,600†	1,600	6,400	25,600¶
AFC27	6,400	6,400	< 1,600	25,600¶
18032460	25,600†	1,600	6,400	6,400
18032404	25,600†	1,600	6,400	25,600¶
Woods	25,600†	1,600	6,400	25,600¶
Citrano	6,400†	1,600‡	1,600§	6,400¶
MAK119	1,600†	1,600	1,600	400
MAK243	6,400	6,400	6,400	25,600¶

*Enzyme-linked immunosorbent assay (ELISA) was performed as described in the Materials and Methods. All serum samples were from mice with the exception of MAK119 and MAK243, which were from humans. The positive cutoff value was at least the average optical density value at 405 nm for negative serum + 2 SD. For mouse serum samples, the cutoff was value 0.1. For MAK119 and MAK243, the cutoff values were 0.1 and 0.04, respectively.
†Showed highest titers with Kpr56.
‡Showed highest titer with Ktr56.
§Showed highest titer with Gmr56.
¶Showed highest titers with TAr56.

TABLE 3
ELISA titers of 14 strain-specific serum samples against chimeric r56 proteins of *Orientia tsutsugamuchi**

Strain†	Antigen used		
	C1	C2	C3
Karp	6,400‡	1,600	1,600
Kato	1,600‡	1,600§	1,600¶
Gilliam	25,600‡	6,400	25,600¶
TA763	25,600‡	25,600§	25,600¶
TH1811	6,400‡	6,400§	6,400¶
TH1812	6,400‡	6,400§	6,400¶
TH1814	25,600‡	25,600§	6,400
AFC27	6,400‡	6,400§	6,400¶
18032460	25,600‡	6,400	6,400
18032404	25,600‡	6,400	25,600¶
Woods	25,600‡	6,400	25,600¶
Citrano	6,400‡	6,400§	6,400¶
MAK119	1,600‡	1,600§	400
MAK243	25,600‡	6,400	25,600¶

*Enzyme-linked immunosorbent assay (ELISA) was performed as described in the Materials and Methods. All serum samples were from mice with the exception of MAK119 and MAK243, which were from humans. The positive cutoff value was at least the average optical density value at 405 nm for negative serum + 2 SD. For mouse serum samples, the cutoff was value 0.1. For MAK119 and MAK243, the cutoff values were 0.1 and 0.04, respectively.
†Strains in **bold** are those with the same titers for at least one of chimerics and the parent Kpr56 and/or TAr56.
‡Showed highest titers with C1.
§Showed highest titers with C2.
¶Showed highest titers with C3.

the chimeric r56 toward any heterologous strains (Tables 2 and 3). Furthermore, for most tested serum samples with the exception of Karp, TA763, TH1811 and TH1812, all chimeric r56s were as reactive as either Kpr56 or TAr56. These results suggested that although the variable domain sequence was modified on the basis of the parent 56-pkD protein, these chimerics still retained similar seroreactivity with most serum samples and could substitute both Kpr56 and TAr56. When comparing the chimerics, C1 appeared to be the best antigen because 14 serum samples had higher titers against C1 than those against C2 and C3. Titers against C1 were as high as those for Kpr56 or TAr56 for seven different serum samples (Table 3).

Although it is difficult to correlate titer against a specific antigen with the overall stimulation of protective immune responses, which consists of humoral and cellular responses, the measurement of titers provides information about the reactivity of certain antigens to antibodies. Thus, it is plausible that these chimerics can be used as reagents for diagnostic purposes and as vaccine candidates. Careful examination of data presented in Tables 2 and 3 shows that inclusion of C1 and Ktr56 is the best combination that may provide the most reactive mixture of r56s with high titers (minimum = 1,600) against all strains evaluated. This combination may provide vaccine candidates that cover the broadest strain variation and have high reactivity for future vaccine candidate evaluation.

DISCUSSION

Cross-reactivity of chimeric protein antigens with serum samples from mice challenged individually with various strains of *Orientia* were analyzed. Titers of these serum samples against the recombinant 56-kD protein of Karp, Kato, Gilliam, and TA763 strains were also compared. These newly made chimeric proteins exhibited similar reactivity with different strain-specific serum samples as the parent proteins.

Furthermore, these chimeric antigens were recognized readily by 14 disparate strains of *Orientia* and they appeared cross-reactive with more strains than Ktr56 and Gmr56. Therefore, one of the three chimerics, particularly C1, can be used as a substitute for the parent proteins for use in diagnosis of *Orientia* infection and as a vaccine candidate to increase the broad protective efficacy. A similar approach can be used to generate more chimerics based on 56-kD protein sequences from different strains of *Orientia* to minimize the number of proteins included in the final vaccine formula and still provide a broad protection against a wide range of strains.

Authors' addresses: Chien-Chung Chao, Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD. E-mail: chien-chung.chao@med.navy.mil. Erin S. Huber, Terrisita B. Porter, Zhiwen Zhang, and Wei-Mei Ching, Viral and Rickettsial Diseases Department, Infectious Diseases Directorate, Naval Medical Research Center, Silver Spring, MD and Uniformed Services University of the Health Sciences, Bethesda, MD, E-mails: ehuber@jhsph.edu, kevin.porter@med.navy.mil, zhiwen.zhang@med.navy.mil, and wei.ching@med.navy.mil.

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